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Restriction and Modification in Group N Streptococci: Effect of Heat on Development of Modified Lytic Bacteriophage†

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The appearance of lytic bacteriophage against newly introduced starter strains used during commercial cheese manufacture occurs rapidly, and their origin is not well understood. In this study, members of the group N streptococci were examined for the presence of bacteriophage restriction and modification systems. Two streptococcal phages from *Streptococcus cremoris* TR and *Streptococcus lactis* C2 (phage designations tr and c2) showed restricted lytic development on *S. cremoris* 799 and KH, respectively. Efficiency of plaquing was 1.9×10^{-7} for tr plaqued on 799 and 2.1×10^{-7} for c2 plaqued on KH. After passage through the restrictive hosts, these phages demonstrated high lytic ability for formerly restrictive hosts. Stress of the restrictive host strains at temperatures of 40 to 50°C resulted in a significant increase in the efficiency of plaquing of restricted bacteriophages. Elevated temperatures are encountered during commercial cheese manufacture. The results suggested that the temporary loss of host restriction activity with the resulting modification of nonspecific bacteriophage may contribute directly to the appearance of lytic phage against new starter strains.

Bacteriophage infection of starter cultures is considered to be the most important factor resulting in slow acid production in large-scale dairy fermentations. Control of bacteriophage lysis of starter cultures has been attempted through rotation of phage-unrelated strains (12), use of phage-inhibitory bulk culture media (11, 28), use of concentrated starter cultures (4), and selection and use of bacteriophage-insensitive (5, 14) or -resistant (16, 31) mutants. These methods are directed toward minimizing bacteriophage proliferation during milk fermentations. However, lytic bacteriophage of undetermined origin continue to appear against new strains introduced into cheese plants.

Spontaneous induction of temperate phage from lysogenic starters has been postulated as a mechanism for the appearance of phage in cheese plants (8, 17). Artificial induction of temperate phage with ultraviolet light or mitomycin C has revealed that lysogeny in the lactic streptococci is common (8, 10, 17, 20, 21, 24, 27). Of 12 commercial mixed starters examined by Park and McKay (24), 9 were found to contain at least one lysogenic strain. Morphological similarities found between artificially induced temperate phage and lytic phage isolated from cheese plants have supported this hypothesis (6). However, a limited number of indicator strains have been identified for lactic streptococcal temperate phage. This may be due partly to the induction of defective phage particles (6, 9, 17) or to the inattentiveness to special conditions that may be necessary to observe plaque formation (33). However, inducible temperate bacteriophages that rarely show lytic ability have questionable significance when the origin of bacteriophage in cheese plants is considered.

Host-controlled restriction of nonspecific bacteriophages has been identified as a common bacterial defense mechanism that functions by recognition and degradation of unmodified deoxyribonucleic acid (1, 2, 18, 22). Phage which escape cellular restriction are modified during lytic development to produce progeny phage that are immune to restriction by that host bacterium. In this manner, highly lytic phage can be produced against bacterial strains previously insensitive to the phage.

Although bacteriophage restriction has been reported in the group N streptococci, few investigators have considered the role of bacteriophage host restriction and modification in the appearance of lytic bacteriophage during milk fermentations. Collins (3) first described host restriction and modification systems in *Streptococcus cremoris* strains in 1956. Restricted bacteriophages were modified to highly virulent particles after one passage through a restrictive host. This increase in phage virulence was not accompanied by alterations in phage adsorption. Restriction and modification of heterospecific

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bacteriophage by group N streptococci have been reported primarily in *S. cremoris* strains (14, 25, 33). Efficiency of bacteriophage infection has been found to vary with the growth stage of the streptococcal host (17, 33). Specifically, an increase was observed when stationary, rather than logarithmic, cells were used to seed bacterial lawns. Additionally, limiting lactose concentrations used in the plaque assay medium also affected plaquing efficiencies (33). These investigations suggest that the nutritional and physiological states of the lactic streptococci may be important factors in the development of restricted phage. Pearce (25) also reported a restriction and modification system operating for a *Streptococcus lactis* ML3 phage (ϕ643) plated on *S. cremoris* KH. Heating starved *S. cremoris* KH cells resulted in a significant decrease in cellular restriction. However, the resulting modified phages showed slow multiplication rates upon adaptation to *S. cremoris* KH as a host strain; therefore, it was suggested that the modified bacteriophage would not become established in commercial cheese-making environments.

In this study, restriction and modification systems were identified in two unrelated strains of *S. cremoris*. Loss of cellular restrictive ability in actively metabolizing cells was accomplished by heat challenge at temperatures of 40 through 50°C. The resulting modified phages also showed high lytic ability on previously restrictive hosts. A study of these systems demonstrated that cellular stress encountered during cheese making may lead to a temporary loss of host restriction, resulting in the appearance of lytic bacteriophages.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. *S. lactis* C2, *S. lactis* ML3, *S. cremoris* KH, and their respective phages (designated c2, ml3, and kh) were obtained from L. L. McKay, Department of Food Science and Nutrition, University of Minnesota, St. Paul. *S. cremoris* TR and 799 and their respective phages, tr and ϕ799, were received from the National Collection of Dairy Organisms, Shinfield, Reading, England (compliments of E. I. Garvie, National Institute for Research in Dairying). All bacterial strains were propagated at 30°C in M17 broth (32). Frozen stock cultures were maintained at −76°C in autoclaved 11% nonfat dry milk solids. Before experimental use, cultures were transferred twice in M17 broth and stored at 4°C. Bacteriophage stocks were prepared in M17 broth as previously described (32) and maintained at 4°C with phage titers approximating 10^9 plaque-forming units (PFU) per ml.

Phage multiplication: temperature parameters. Five-hour cultures propagated in M17 broth at 30°C were dispensed (0.3 ml) into small sterile test tubes containing 0.05 ml of 1 M $CaCl_2 \cdot 7H_2O$. Immediately after the cells had been heated for 2 min at the experimental temperature, 0.1 ml of the appropriate phage suspension was added. Heating of the phage-cell suspension continued for the specified time, and the tubes were then placed at 30°C for 10 min to allow for low-temperature phage adsorption. After the adsorptive period, the samples were assayed for plaque formation (32).

Bacteriophage adsorption. Bacteriophage suspensions were diluted to approximately 10^5 PFU/ml in 10% M17 broth, and 1 ml was added to a sterile centrifuge tube. A 1-ml amount of bacterial cells (5-h culture, 30°C) and 0.05 ml of 1 M $CaCl_2 \cdot 7H_2O$ were added, followed by gentle mixing. After incubation for 15 min at room temperature, the suspension was centrifuged at 9,750 × *g* for 10 min to remove bacterial cells and adsorbed phage. The supernatant fluid was assayed immediately for residual phage by standard plaque assay on a sensitive host. Phage adsorption was calculated as follows: percent adsorption = [(control titer − residual titer)/control titer] × 100.

Lytic development of modified bacteriophage. Single-plaque isolates were obtained from low-efficiency phage crosses on restrictive hosts. Plaques were picked into 1 ml of M17 broth (using sterile, plugged Pasteur pipettes), blended in a Vortex mixer, and stored overnight at 4°C. Single-plaque isolates were propagated once on the restrictive hosts for use in the following experimental procedures. To determine the lytic activity of the modified phage on broth cell cultures, the phage suspension was added to 10 ml of M17 broth containing 0.05 ml of 1 M $CaCl_2 \cdot 7H_2O$ in a sterile cuvette. The optical density at 650 nm was adjusted to 0.1 with 0.1-ml samples of a 6-h cell culture of the previously restrictive host. The optical density was followed for 6 h of bacterial growth at 30°C with a Bausch & Lomb Spectronic 70 spectrophotometer.

Activity test to determine generation of modified phage. The activity test used to determine the generation of modified phage was a modification of that described by Heap and Lawrence (5). The unmodified phage preparation (0.2 ml) was added to 0.2 ml of an overnight milk culture of the appropriate restrictive host (tr phage with 799 cells and c2 phage with KH cells). This mixture was gently blended in a Vortex mixer, and the phage was allowed to adsorb at room temperature for 7 min. Eleven percent reconstituted nonfat dry milk solids (9.6 ml, steamed for 60 min) was then added to the starter culture-phage mixture. This suspension was incubated at 30°C for 70 min and then at 40°C for 190 min and returned to 30°C for 100 min.

After incubation, each milk sample was coagulated with 2.5 ml of sterile 10% lactic acid and centrifuged at 9,750 × *g* for 15 min. The resulting whey was adjusted to pH 7.0 with sterile 1 N NaOH, centrifuged at 9,750 × *g* for 15 min, and filtered through a 0.45-μm filter (Acrodisc, Gelman Instrument Co.). The whey was then assayed for plaque formation as previously described.

RESULTS

Bacteriophage adsorption and restriction. Previously, Oram and Reiter (23) studied

the adsorptive and replicative abilities of lactic streptococcal bacteriophage on six strains of *S. cremoris* and *S. lactis*. These phage-host interactions indicated that each bacteriophage was specific in its replication pattern, but had a less restricted range of adsorption. The insensitivity of strains to adsorbing phage suggested to us that restriction systems may be involved as a host defense. Results of heterospecific phage crosses on five streptococcal strains are shown in Table 1. In *S. cremoris* KH and 799, restricted lytic development was observed for phages c2 and tr, respectively. Plaques formed during these restrictive crosses were smaller and more irregular than those which were observed on sensitive host strains. The smaller plaque size may have contributed to the previous inability to observe low-efficiency crosses on *S. cremoris* KH and 799, due to poor plaque development on media other than M17 (32). *S. lactis* C2 and ML3 appear to be very closely related strains as both c2 and ml3 phages plaqued at equal efficiency on either strain. However, only c2 phage, not ml3 phage, was capable of low-efficiency lytic development on *S. cremoris* KH.

Bacteriophage adsorption was examined to determine whether plaque efficiency varied with phage adsorptive capability (Table 2). In all cases, phage adsorption to original host strains exceeded 90%. Of the restrictive crosses, phage tr adsorbed equally well to its original host and restrictive host (*S. cremoris* 799), whereas c2 phage showed only 54% adsorption to *S. cremoris* KH. Only 13% of the ml3 phage population adsorbed to *S. cremoris* KH. Although 13% adsorption indicates a large number of successfully adsorbed phage when the high-titer phage preparations used are considered, this adsorption level may be low enough to account for the inability to observe plaque formation by ml3 phage on *S. cremoris* KH. Pearce (25) reported 31% adsorption and an efficiency of plaquing (titer on restrictive host + titer on original host) of 10^{-5} for an *S. lactis* ML3 phage (ϕ643) propagated on *S. cremoris* KH. The variability in adsorption of c2, ml3, and ϕ643 phages to *S. cremoris* KH suggests that there are structural differences in these phages that may account for the presence or absence of low-efficiency plaque formation. Differences in adsorption and plaquing efficiency by phage ml3 on *S. cremoris* KH, when compared with data reported for ϕ643 (25), indicate that the ml3 phage used in this study differed from the *S. lactis* ML3 lytic phage ϕ643 previously employed by Pearce (25). In *S. cremoris* KH and 799, the level of adsorption shows that restricted development of c2 and tr phages occurs at a point beyond phage adsorption. These data indicate that operational restriction and modification systems are present in *S. cremoris* KH and 799.

TABLE 1. *Efficiency of plaque formation among selected lactic streptococci*

Phage[a]	Host strain	Efficiency of plaquing[b]
ml3	*S. cremoris* KH	0
ml3	*S. lactis* C2	0.7
c2	*S. cremoris* KH	2.1×10^{-7}
c2	*S. lactis* ML3	1.0
tr	*S. cremoris* 799	1.9×10^{-7}
799	*S. cremoris* TR	0

[a] Phage titers on host strains exceeded 10^9 PFU/ml.
[b] Titer on restrictive host + titer on original host.

TABLE 2. *Adsorption of bacteriophage to original and restrictive hosts*

Strain	Phage	Adsorption (%)[a]
S. cremoris KH	c2	54
S. cremoris KH	ml3	13
S. lactis C2	c2	92
S. lactis ML3	ml3	98
S. cremoris TR	tr	99
S. cremoris 799	tr	99

[a] Calculated as the percentage remaining of the control titer.

Properties of modified bacteriophages. Plaque isolates from restrictive crosses were titrated on original and restrictive hosts to determine host-specific modifications occurring in the bacteriophages (Table 3). Phage c2, once propagated through *S. cremoris* KH (designated mc2·KH), showed equal plaquing ability on the original host, *S. lactis* C2, and on the restrictive host, *S. cremoris* KH. Therefore, the *S. cremoris* KH modification is not recognized in *S. lactis* C2. Previously, the *S. cremoris* KH modification of ϕ643 was also shown not to be recognized by *S. lactis* ML3 (25). Once passed through *S. lactis* C2, mc2 phage was again restricted by *S. cremoris* KH. Conversely, once passed through *S. cremoris* 799, phage tr was restricted by *S. cremoris* TR and plaqued at high efficiency on *S. cremoris* 799. Therefore, the 799 modification of the tr phage was recognized by *S. cremoris* TR. After a single passage through *S. cremoris* TR, the 799 modification was lost, and the tr phage was again restricted by *S. cremoris* 799.

To determine the lytic ability of the modified bacteriophages on restrictive hosts, lysis of *S. cremoris* 799 and KH cells in broth culture was examined (Fig. 1). Phages tr and c2 were unable to slow cell growth of the restrictive hosts. After passage through the restrictive hosts, modified

phages mtr·799 and mc2·KH were capable of lytic activity on the restrictive hosts at a level that stopped cell growth in broth culture. The presence of the modified phage in milk cultures of *S. cremoris* 799 or KH effectively inhibited normal acid development by these strains (data not shown). The results demonstrated that the modified bacteriophages can achieve high lytic ability on previously restrictive hosts at levels sufficient to inhibit normal starter culture activity.

Effect of temperature on bacteriophage restriction. Temporary inactivation of host restriction systems by elevated temperature has been reported in *Staphylococcus aureus* (30), *Escherichia coli* (29), and *Salmonella* species (34). In *S. cremoris* KH, inactivation of restrictive activity against phage ϕ643 was accomplished within 10 min at 40°C (25). However, inactivation of restrictive ability first required starvation of the cells before heat exposure. To determine the heat susceptibility of restriction activity in *S. cremoris* 799 and KH, actively metabolizing cells contained in M17 broth were challenged with phages tr and c2 and heated for 10 or 30 min at temperatures ranging from 30 to 50°C (Fig. 2). Little loss of phage restriction was observed between 30 and 40°C for either strain. However, a 3- to 5-log increase was observed in the efficiency of plaquing of tr and c2 phages when heated to 50°C for 10 or 30 min. Heating of cells for longer than 10 min did not substantially increase the loss of restriction, indicating that, at temperatures greater than 40°C, loss of activity occurs soon after reaching these temperatures. However, at 40°C, the length of heat treatment did affect the loss of restrictive activity in both systems (Fig. 3). Initially, bacteriophage restriction increased slightly, but extended heating at 40°C for an additional 45 min increased the efficiency of plaquing by 1 log cycle to values approaching 10^{-6}. Although the loss of restriction was not as great as that which was observed at higher temperatures, the results do indicate that prolonged heating of *S. cremoris* KH or 799 at 40°C contributes to the loss of phage restriction.

TABLE 3. *Plaquing ability of bacteriophage isolates propagated on modified and restrictive hosts*

Phage	Host	PFU/ml
mc2·KH[a]	*S. lactis* C2	3.0×10^5
	S. cremoris KH	3.0×10^5
mc2·KH·C2[b]	*S. lactis* C2	2.6×10^7
	S. cremoris KH	$<1 \times 10^1$
mtr·799	*S. cremoris* TR	3×10^1
	S. cremoris 799	3.3×10^5
mtr·799·TR	*S. cremoris* TR	1.6×10^7
	S. cremoris 799	1.8×10^2

[a] One passage of c2 phage through *S. cremoris* KH was carried out before plaque assay on the indicated hosts. All plaque isolates were titrated directly on host strains.

[b] Sequential passage of c2 phage through *S. cremoris* KH and then *S. lactis* C2 was carried out before plaque assay on the indicated hosts.

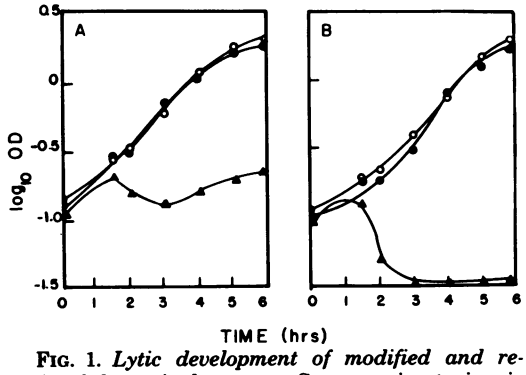

FIG. 1. *Lytic development of modified and restricted bacteriophages on S. cremoris strains in broth culture. (A) Growth of S. cremoris 799: (●), control cells; (○), cells in the presence of phage tr; (▲), cells in the presence of the modified tr phage. (B) Growth of S. cremoris KH: (●), control cells; (○), cells in the presence of phage c2; (▲), cells in the presence of the modified c2 phage. OD, Optical density.*

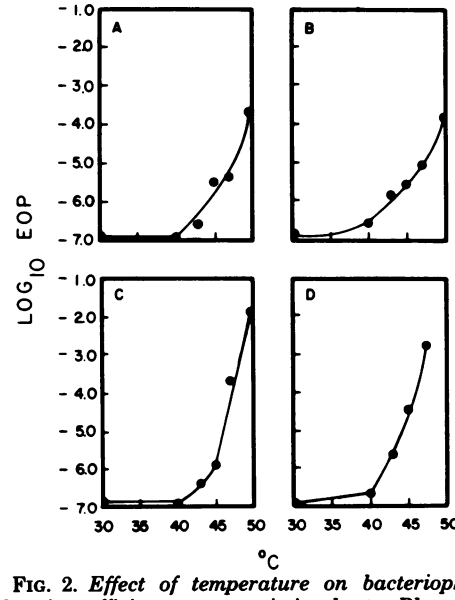

FIG. 2. *Effect of temperature on bacteriophage plaquing efficiency on restrictive hosts. Phage-cell suspensions were heated for either 10 min (A and C) or 30 min (B and D) at the indicated temperature. (A and B) Phage tr on S. cremoris 799; (C and D) Phage c2 on S. cremoris KH. EOP, Efficiency of plaquing.*

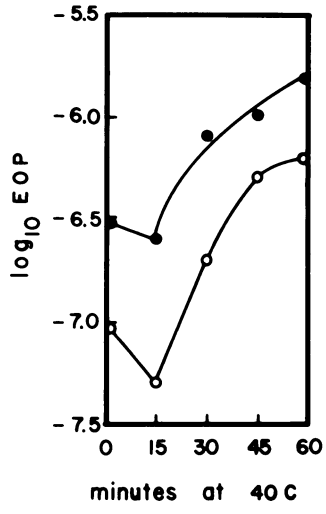

FIG. 3. *Effect of extended heat exposure at 40°C on plaquing efficiencies of phage tr on S. cremoris 799* (○) *and of phage c2 on S. cremoris KH* (●). *EOP, Efficiency of plaquing.*

Appearance of modified bacteriophage. Heap and Lawrence (5), attempting to select for starter strains that remain insensitive to existing bacteriophages, developed a laboratory activity test that accurately predicted the appearance of lytic bacteriophages active against strains introduced into New Zealand cheese factories. Strains were propagated repeatedly through milk in the presence of nonspecific phage to determine the number of growth cycles that could be achieved before a lytic phage would appear against the strain tested. Phage-insensitive strains could be propagated through eight growth cycles without the appearance of a lytic bacteriophage. Alternatively, lytic phage would appear against susceptible strains within one growth cycle through the activity test. To determine whether modified lytic phages would rapidly appear against the restrictive hosts under the conditions of the activity test, phages tr and c2 were added to inoculated milk cultures of *S. cremoris* 799 and KH, respectively. Initially, whey samples assayed from the milk cultures showed less than 1 PFU/ml for phages tr and c2 on each of their restrictive hosts. After a single growth cycle, lytic phage populations against *S. cremoris* 799 and KH were detected in the whey samples at levels of 5.6×10^4 and 6.3×10^4 PFU/ml, respectively. Lytic phage against either *S. cremoris* strain were not detected in whey samples from coagulated milk cultures that did not receive the 40°C heat treatment. The results show that restricted bacteriophage can develop rapidly into a substantial lytic phage population under laboratory conditions established to mimic starter culture activity in a commercial cheese-making operation.

DISCUSSION

We report operational evidence for the presence of restriction and modification systems in two strains of *S. cremoris*. More importantly, exposure of *S. cremoris* 799 and KH to temperatures equal to or exceeding 40°C resulted in a reduction of phage restriction, leading to the appearance of lytic phage modified for efficient multiplication on the new host. Pearce (25) reported the presence of a temperature-dependent restriction and modification system in starved *S. cremoris* KH cells challenged with an *S. lactis* ML3 phage. Because an increased plaquing efficiency of restricted phage was only seen in starved cells and the modified phage replicated slowly, the author concluded that the establishment of the modified phage in a commercial cheese-making environment would be unlikely. During this study, the identification of heat-susceptible restriction systems in actively metabolizing starter strains emphasizes the possible importance of this mechanism in the appearance of lytic bacteriophages in cheese plants.

A study of bacteriophage restriction in the group N streptococci suggested that nonspecific phage infection may be dependent on the growth and nutritional state of the cell. For example, Lowrie (17) reported the induction of a temperate bacteriophage from *S. cremoris* R1 that showed lytic activity on *S. cremoris* AM1. The plaquing efficiency for this phage increased considerably when stationary, rather than logarithmic, cells were used to seed bacterial lawns. Also, Terzaghi and Terzaghi (33), apparently using the identical system defined previously by Lowrie (17), reconfirmed the loss of bacteriophage restriction that accompanied cell transistion to stationary growth. However, Collins (3) did not observe an appreciable difference in restricted plaque formation between young and old cultures of *S. cremoris*. The growth state of the cell and the effect on nonspecific phage replication are important considerations when streptococcal strains for cross-reacting bacteriophages are typed. Decisions on strains that are paired or rotated through commercial starter culture programs are strongly influenced by phage host range (11, 13); therefore, any limitations imposed via the testing conditions to detect phage lytic ability, at any level, should be eliminated (33). However, when the appearance of lytic phage during milk fermentations is considered, attention must be focused on the restrictive ability of the cell during active growth. Under conditions

of active starter fermentation in a fluid milk system, stationary-phase cells would not predominate; therefore, the loss of restriction in these cells should not contribute to the appearance of modified lytic bacteriophage in cheese plants.

Bacteriophage populations can rapidly achieve high levels upon successive use of starter strains in large cheese-processing operations. In the presence of high phage populations, the effect of low-efficiency phage replication on normally restrictive hosts can be dramatic. The dynamics of phage replication indicate that a single burst of modified phage could rapidly lead to the appearance of a substantial lytic phage population. In addition, any treatment that increases the rate of release of modified lytic phage would also increase the likelihood of starter culture failure. The process of cooking cheese curd at 40°C for times equal to or exceeding 1 h, with requirements for subsequent starter growth and acid production, establishes ideal conditions for the temporary breakdown of cellular restriction. In this study, modified lytic phage populations (approximating 5×10^4 PFU/ml) appeared against both *S. cremoris* strains after one growth cycle through milk during the starter culture activity test. Undoubtedly, the heat treatment included during the activity test facilitated the loss of restriction, leading to the development of a modified lytic phage population.

Alternatively, the exposure of lactic streptococci to elevated temperatures during cheese making may contribute to the loss of cellular restriction by an additional mechanism. The distribution of plasmid deoxyribonucleic acid throughout members of the group N streptococci has been well established (19). Extrachromosomal elements have been correlated with fermentative metabolism through direct linkage of plasmid species to both lactose utilization and proteinase activity (19). Furthermore, variability in phage sensitivity that accompanied the appearance of slow-acid variants in the lactic streptococci suggested that host restriction enzymes may also be linked to plasmid deoxyribonucleic acid (15). Plasmid determinants for restriction and modification systems have been reported in numerous bacterial systems (26), and considering the multiplasmid nature of the lactic streptococci, it seems probable that plasmids may play a direct role in the restrictive activities of these bacteria. If restriction enzyme systems in the lactic streptococci are linked to plasmid deoxyribonucleic acid, then growth at elevated temperatures may generate restrictionless variants due to plasmid loss. High-temperature destabilization of plasmids has been commonly used to generate cured variants. Additionally, Holloway (7) observed that a restriction and modification system in *Pseudomonas aeruginosa* was lost after three generations at 43°C and could not be regained upon subsequent culture transfer, implicating the involvement of plasmid deoxyribonucleic acid. The loss of restriction plasmids during growth at elevated temperatures could generate cell variants susceptible to nonspecific bacteriophage. Assuming that modification enzymes are not lost concurrently with the restriction activity, the progeny of phage replication in these variants would provide an additional source for the appearance of modified, lytic bacteriophage during dairy fermentations.

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